Detailed report on the first European inter-laboratory comparison study on the determination of acrylamide in butter cookies and crispbread

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Introduction

Acrylamide (AA) is a substance that has found widespread application in industry, e.g. for the purification of drinking water and in food packaging. Due to its toxicological properties, legal limits have been set for both drinking water and for migration into food [1-5].

Since the finding of elevated levels of acrylamide in heat-treated potato products and other goods was reported by the Swedish National Food Authority in April 2002, concerted efforts have been made to try to improve the image of the nutritional uptake of this substance by monitoring its content in different kinds of food [6, 7].

Following a request of the participants of the European workshop on "Analytical methods for the acrylamide determination in food" (April 2003, Oud-Turnhout, Belgium), the Institute for Reference Materials and Measurements (IRMM) of the European Commission's Directorate General Joint Research Centre (DG JRC) organised an inter-laboratory comparison test on the determination of AA in butter cookies and crispbread samples [8]. One goal of the collaborative trial was the evaluation of the performance of laboratories at an AA level close to the limit of quantification (LOQ). Another goal was the elucidation of the most critical steps in the applied analysis protocols. For that purpose, a raw bread extract and a spiked bread extract were shipped with the food samples. The set of samples was completed by AA standard solutions, which were prepared by dissolution of solid AA in appropriate solvents.

The study was a dedicated collaborative trial and was free of charge for the participants. It was announced via the Directorate General Health and Consumer Protection (DG SANCO) to the national food authorities of EU Member States and EU Candidate Countries. Additionally all participants of the above-mentioned workshop were informed by email. Information concerning the application procedure for the study was also available on the homepage of the Food Safety and Quality Unit (FSQ) of IRMM.

In order to facilitate the application procedure, a special application form was sent to interesting laboratories (see Annex 1).

A number of 78 laboratories applied for the participation in the ring trial and was supplied with test samples. Most laboratories belonged to 14 European countries. One laboratory was located in Canada. The receipt of the test samples had to be confirmed by the participants via the sample receipt form (see Annex 2).

The participants were asked to determine the AA content in the test samples by application of their in-house analysis methods.

62 data sets with the analysis results of at least one sample were reported to the organisers of the study in applying a special report form that was created and sent to the participants for that purpose (see Annex 3).

Test Materials

Commercial brands of butter cookies and crispbread were purchased in German local markets. The butter cookies were ground with an Alexanderwerk mill (Alexanderwerk Inc., Horsham, PA, USA) and tumbled for 1 h using a cement mixer. Afterwards the powder was finely ground using a Retsch Z1 mill (2 mm screen pack) (Retsch GmbH &Co. KG, Haan, Germany). The resulting powder was homogenised in a cement mixer for 1 h and finally sieved (mesh size 0.85 mm). The crispbread was coarsely ground with a Romer Analytical Sampling Mill (Romer Labs Inc., Union MO; USA) before subsequent grinding with a Baumeister UDL VA mill (1 mm hole screen) (Baumeister Verfahrenstechnik GmbH, Norderstedt, Germany). The resulting powder was homogenised in a cement mixer for 1 h. Both materials were split into portions of approximately 50 g in vacuum-packed aluminium-clad bags, which were stored at +4 °C. Each bag was individually numbered.

Raw and spiked white bread crumb extracts

White bread from a local bakery was cut into slices, the crust was removed and the remaining crumb was cut into cubes of about 1.5 cm of length of each side. 1216 xg of the crumb was weighed into a 15 L bucket. 12 L of water was poured over the crumb cubes, which were extracted by maceration for 1 h at room temperature. The extract was filtered through cotton wool for medical purposes. The milky extract was divided into two equal portions. One portion remained untreated and was filled into 50 mL brown glass ampoules, while the other portion was spiked with an aqueous AA standard solution to give a spiking level of 118 ng/mL. The spiked extract was homogenised by intensive stirring and was also filled into 50 mL brown glass ampoules. To avoid additional distortion of the matrix, neither the raw extract nor the spiked extract were stabilised. All ampoules were filled close to the rim, were tightly sealed with PTFE coated butyl septa in aluminium crimp caps and were labelled with self-adhesive paper labels that contained the sample name and a short sample description.

Acrylamide standards solutions

The standard solutions were prepared by careful weighing of about 20 mg acrylamide of minimum 99 % purity (Sigma, Sigma-Aldrich CO, St. Louis, MO, USA) into 100 mL volumetric flasks and dissolved in high purity water (MilliQ, Millipore, Brussels, Belgium) or ethyl acetate (EtAc), quality SupraSolv^{TM}, (Merck, Darmstadt, Germany) for GC/MS measurement without derivatisation of AA. The standards were diluted to give a final AA concentration of 48.2 ng/mL for the aqueous solution and 149.5 ng/mL for the EtAc. 25 mL ampoules were filled with the standard solutions and tightly sealed with PTFE coated butyl septa and aluminium crimp caps. The ampoules were labelled as "Acrylamide standard" with the solvent name in brackets.

All filled ampoules were put immediately into a refrigerator and were stored at 4 °C.

Dispatch of samples

All samples were sent via express mail in polystyrene boxes, equipped with a cooling cell, which was precooled at -20 °C. Most of the participants in countries of the Schengen treaty reported sample receipt within 24 hours after sending. A few laboratories had to be supplied with a second set of samples due to the break of ampoules during delivery, respectively improper storage of the packages at the customs followed by sample degradation.

Homogeneity of samples

Homogeneity was tested according to the International Harmonised Protocol for Proficiency Testing of Analytical Laboratories [9].

<u>Crispbread and butter cookies</u>

Ten randomly selected packages of each test sample were analysed in duplicate applying following method: Two g of the homogenised sample was defatted with iso-hexane. Internal standard, d3-acrylamide, (2 µg) was added and after an exposure time of 30°min, 20 mL of water was admixed. Acrylamide was extracted in a sonicator at 60°C for 30 min. The sample was purified by adding 20 mL of acetonitrile and 500 µL of Carrez I (potassium hexacyanoferrat (II), c = 150 g/L) and Carrez II (zinc acetate, c = 300 g/L), respectively. The

sample was centrifuged at 4500 xg for 10 min and the supernatant was filtered through a membrane filter.

The quantification of acrylamide was performed by liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) with electrospray positive ionisation acrylamide was identified by multiple reaction monitoring (MRM) set to records m/z 72>72, 72>55 and 72>44. Monitored transitions for the internal standard were m/z 75>75, 75>58 and 75>44. Quantification was performed by comparison of the peak area ratio of acrylamide with the internal standard d_3-acrylamide, monitored by using the MRM transition m/z 72>55 (acrylamide) and 75>58 (d_3-acrylamide).

Standard and bread extracts

Ten randomly ampoules of each extract were analysed in duplicate by LC/MS/MS applying a modification of the analysis protocol of Rosén and Hellenäs [10]. The modification consisted of the addition of a Carrez precipitation step prior to the solid phase extraction.

The standard solutions were homogenised by vigorously shaking, therefore sufficient homogeneity could be assumed. The AA content of the standard solutions was checked by six-fold LC/MS/MS measurement of the aqueous standard and six-fold GC/MS measurement of the standard in EtAc. Since the standard solutions did not contain any internal standard (ISTD), d_3-AA, 98% deuterium (Cambridge Isotope Laboratories, Andover, MA, USA) was added prior to the measurements.

The homogeneity of the test samples were proved by subjecting the results of the duplicate measurements to one-way “analysis of variance” (ANOVA). The results are given in Table 1-3 of Annex 4. Sufficient homogeneity was found for the butter cookies sample, the crispbread sample and the spiked bread extract. Homogeneity of the raw extract could not be evaluated due to results of analysis below the LOQ of the applied LC/MS/MS method (5 ng/mL).

Statistical evaluation of the results

Assigned value

The assigned concentration of AA in the test materials was calculated for the respective test sample from the reported mean values of the duplicate determinations of the participants by application of robust statistics. The striking advantages of robust statistics compared to the traditional approach has recently been demonstrated by M. Thompson [11]. It has the advantage that the detection and rejection of outliers is not necessary thus, the impact of extreme values on the average and the standard deviation is down weighted. Furthermore, it works well with data distributions that deviate significantly from normal distribution, as it was the case in this study. The robust mean values and robust standard deviations were computed by application of a MS Excel® macro that was written by the Analytical Methods Committee of The Royal Society of Chemistry (AMC). The respective figures are tabulated for each test sample in the following sections of the report.

Performance indicator and target standard deviation

The performance of the respective laboratory is expressed by the z-score, which is calculated according to equation 1.

$$z_i = \frac{x_i - \overline{X}}{\sigma} \qquad \text{Equation 1}$$

z_i: z-score of laboratory i for the respective sample; x_i reported AA content of laboratory i for that sample, expressed as the mean of duplicate determinations; $\overline{X}$: assigned value for the respective sample, σ: target standard deviation

The target standard deviation was calculated according to a proposal of M. Thompson, which applies a concentration dependent modification of the Horwitz equation [12]. Below an assigned value of 120 µg/kg, the target standard deviation was set to 22 % of the assigned value. Above that border value, it was calculated according to equation 2, which includes the assigned value, expressed as dimensionless mass ratio (1 µg/kg ~ 1 ppb = 1.10^{-9}).

$$\sigma = 0{,}02\frac{\left(\overline{X} * 1.10^{-9}\right)^{0{,}8495}}{1.10^{-9}} \qquad \text{Equation 2}$$

σ: target standard deviation; $\overline{X}$: assigned value (µg/kg),

Since the target standard deviation depends only on the assigned value, it is not influenced by the width of the distribution of the reported analysis results. Consequently, the comparison of different proficiency tests (PTs) on the same analyte/matrix combination is facilitated.

z-Scores were calculated for the crispbread sample, the butter cookies sample and the spiked bread extract. They were not computed for the AA standard solutions, because this would not reflect the laboratories proficiency in the determination of AA in food. The acceptability of the laboratory performance was evaluated according to the following limits [9]:

$\lvert z\rvert \leq 2.0$	satisfactory
$2.0 < \lvert z\rvert < 3.0$	questionable
$\lvert z\rvert \geq 3.0$	unsatisfactory

A z-score was not assigned, if the reported AA content was below the LOQ.

Evaluation of the analysis data for the crispbread sample

Overview

A number of 50 laboratories reported real figures for the AA content of the crispbread sample. The residual 12 stated an AA content below their LOQ. The lowest result was 15.5 µg/kg, the highest 668 µg/kg. The distribution of the analysis results was not symmetric. It showed a sharp cut-off at 30 µg/kg, which was traced back to the LOQs of the applied methods that were mostly communicated between 30 µg/kg and 50 µg/kg.

Assigned value and target standard deviation

The assigned value was determined by different procedures, all comprising robust statistics. The simplest robust estimate of the mean value is the median. A more elaborated estimation is represented by an iterative approach that is known as Huber H15. The median was determined to 57 µg/kg, while the Huber H15 estimate was 65.5 µg/kg. This large deviation between the two values could not be neglected and gave therefore reason for the application of alternative methods for the determination of the assigned value.

The first approach consisted of the exclusion of laboratories that reported AA contents of the standard solutions outside the range of ± 15 % of the calculated value. The median of the residual 23 laboratories was 53.5 µg/kg, the Huber H15 estimate 54.3 µg/kg.

Another attempt consisted of the calculation and evaluation of "running z-scores" (RZ). They were computed from z-scores of former proficiency tests that were questioned with the details of the applied analysis method. The RZ values were determined according to equation 3.

$$RZ = \frac{\sum_{i=1}^{k} z_i}{k} \qquad \text{Equation 3}$$

RZ: running z-score; z_i: z-score of PT test I; k: number of PT tests

A number of 33 participants took part in previous collaborative trials (proficiency tests) for the determination of AA in food. The majority of them participated at least in 2 former tests, 10 communicated the results of 3 up to 5 tests. The results of analysis of laboratories with running z-scores below |1| were selected for an alternative calculation of the assigned value. The median of the results of the 21 laboratories, which met this claim, was 53.5 µg/kg, the Huber H15 estimate 54.8 µg/kg. The best estimate of the mean value seems to lie between

53.5 and 55 µg/kg. However, it should be noted that the majority of the laboratories that were included in the two evaluations was identical. Additionally, both evaluations included not acceptable performance values up to a z-score of about 6. This means that not all laboratories that fulfilled the prerequisites for consideration into these evaluations performed well in the current study.

However, both evaluations confirmed that the median of the whole data set represents the mean value better than the respective Huber H15 value.

For that reason, it was decided to select the median of the whole data set (57 µg/kg) as the assigned value.

Consequently, the target standard deviation was calculated to 12.5 µg/kg.

z-Scores of the participants

The mean values of the duplicate determinations of AA in the crispbread sample are tabulated with the corresponding z-score in table 1. Figure 1 shows the plot of z-scores in ascending order. Laboratories, which are not considered, reported "below LOQ" as result.

Figure 1: Plot of z-scores for the crispbread sample (Lab code: laboratory code)

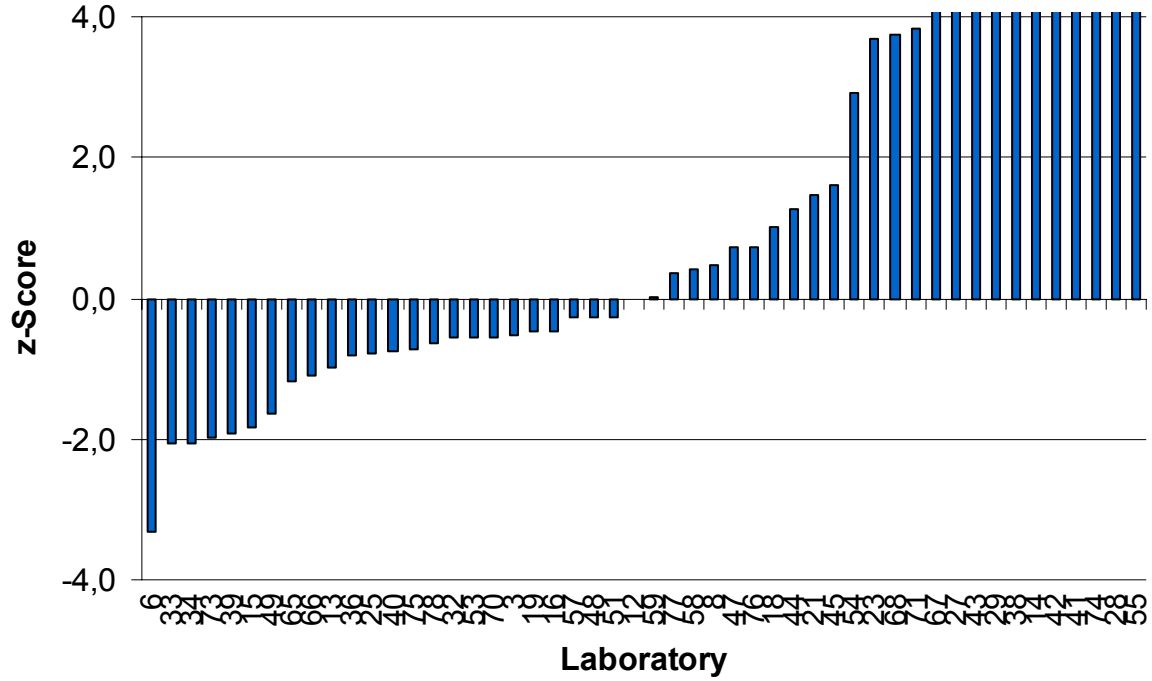

Table 1: Results of analysis and z-scores for the crispbread sample; bold printed z-scores mark unsatisfactory results

Laboratory	Reported result	z-Score	Laboratory	Reported result	z-Score
	µg/kg			µg/kg	
3	50.50	-0.52	**43**	120.00	**5.03**
6	15.50	**-3.31**	**44**	73.00	1.28
8	63.00	0.48	**45**	77.00	1.60
12	56.97	0.00	**47**	66.00	0.72
13	44.50	-1.00	**48**	53.50	-0.28
14	140.00	**6.62**	**49**	36.35	-1.65
15	34.00	-1.83	**51**	53.57	-0.27
16	51.00	-0.48	**53**	50.00	-0.56
18	69.82	1.02	**54**	93.43	**2.91**
19	50.95	-0.48	**55**	668.00	**48.74**
21	75.47	1.47	**57**	53.45	-0.28
23	103.15	**3.68**	**58**	62.00	0.40
25	47.00	-0.80	**59**	57.00	0.00
27	118.25	**4.89**	**65**	42.00	-1.20
28	606.50	**43.83**	**66**	43.29	-1.09
29	121.00	**5.11**	**67**	109.50	**4.19**
32	50.00	-0.56	**68**	104.00	**3.75**
33	31.00	**-2.07**	**70**	50.00	-0.56
34	31.00	**-2.07**	**71**	105.00	**3.83**
36	46.85	-0.81	**73**	32.00	-1.99
38	121.53	**5.15**	**74**	500.00	**35.34**
39	33.00	-1.91	**75**	48.00	-0.72
40	47.55	-0.75	**76**	66.00	0.72
41	398.00	**27.20**	**77**	61.50	0.36
42	198.50	**11.29**	**78**	49.05	-0.63

Evaluation of the analysis data for the butter cookies sample

Overview

A number of 59 analysis results were considered in the data evaluation of the butter cookies sample. Two laboratories reported an AA content below the LOQ of their method, another one did not report any result for that sample. The results of analysis ranged over one order of magnitude. The lowest value was 82.0 µg/kg, the highest 890.5 µg/kg. The distribution of the analysis results is more symmetric than that of the crispbread sample.

Assigned value and target standard deviation

The assigned value was determined in applying robust statistics. For this sample, the median and the Huber H15 estimate of the mean value showed good agreement. The plausibility of the assigned value was checked again by extraction of the analysis results of laboratories with RZ values below |1|. The median of this group was determined to 150.0 µg/kg, the Huber H15 estimate to 151.6 µg/kg. Therefore the median of the whole data set (150.0 µg/kg), including the data of all participants, was chosen as the assigned value.
The respective target standard deviation was calculated according to equation 2 to 31.9 µg/kg.

z-Scores of the participants

The mean values of the duplicate determinations of AA in the butter cookies sample are tabulated with the corresponding z-score in table 2. Figure 2 shows the plot of z-scores in ascending order. Laboratories, which are not considered, reported “below LOQ”, or did not report any result.

Figure 2: Plot of z-scores for the butter cookies sample

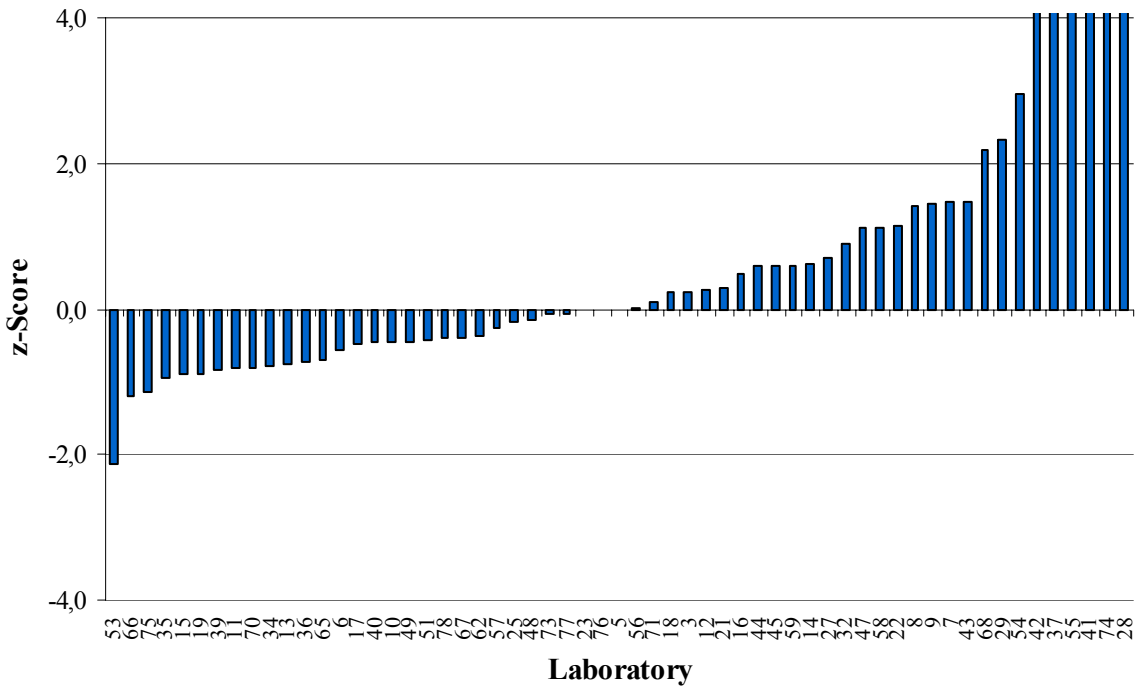

Table 2: Results of analysis and z-scores for the butter cookies sample; bold printed z-scores mark results outside the acceptable range

Laboratory	Reported result	z-Score	Laboratory	Reported result	z-Score	Laboratory	Reported result	z-Score
	µg/kg			µg/kg			µg/kg	
3	157,50	0,23	**27**	172,10	0,69	**54**	244,64	**2,96**
5	150,05	0,00	**28**	890,50	**23,19**	**55**	353,00	**6,36**
6	132,00	-0,57	**29**	224,00	**2,32**	**56**	150,50	0,01
7	197,00	1,47	**32**	179,00	0,91	**57**	142,15	-0,25
8	195,00	1,41	**34**	125,00	-0,78	**58**	186,00	1,13
9	196,00	1,44	**35**	119,50	-0,96	**59**	169,00	0,59
10	136,00	-0,44	**36**	126,50	-0,74	**62**	138,34	-0,37
11	124,50	-0,80	**37**	350,00	**6,26**	**65**	128,00	-0,69
12	158,00	0,25	**39**	123,00	-0,85	**66**	111,67	-1,20
13	125,50	-0,77	**40**	135,60	-0,45	**67**	137,63	-0,39
14	170,00	0,62	**41**	462,00	**9,77**	**68**	219,43	**2,17**
15	121,50	-0,89	**42**	288,00	**4,32**	**70**	124,50	-0,80
16	165,00	0,47	**43**	197,00	1,47	**71**	153,50	0,11
17	134,50	-0,49	**44**	169,00	0,59	**73**	147,50	-0,08
18	157,35	0,23	**45**	169,00	0,59	**74**	550,00	**12,53**
19	121,75	-0,89	**47**	186,00	1,13	**75**	114,00	-1,13
21	159,60	0,30	**48**	145,50	-0,14	**76**	150,00	0,00
22	186,50	1,14	**49**	136,00	-0,44	**77**	147,50	-0,08
23	149,45	-0,02	**51**	136,34	-0,43	**78**	136,95	-0,41
25	144,50	-0,17	**53**	82,00	**-2,13**			

Evaluation of the analysis data for the spiked bread extract

Overview

A number of 60 analysis results were considered in the data evaluation of the butter cookies sample. One laboratory reported an AA content below the LOQ of its method, another one did not report any result for that sample. The results of analysis ranged also for that sample over one order of magnitude. The lowest value was 51.0 µg/kg, the highest 560 µg/kg. The distribution of the analysis results is comparable to that of the butter cookies sample.

Assigned value and target standard deviation

The assigned value was determined in applying robust statistics. For this sample, the median and the Huber H15 estimate of the mean value showed very good agreement. The spiking level was 118 ng/mL. However, since analyte loss due to e.g. irreversible adsorption cannot be excluded, the median of the whole data set (116.3 ng/mL), including the data of all participants, was chosen as the assigned value.

The respective target standard deviation was calculated according to equation 2 to 25.6 ng/mL.

z-Scores of the participants

The mean values of the duplicate determinations of AA in the butter cookies sample are tabulated with the corresponding z-score in table 3. The results of analysis of participants that reported real figures for the raw bread extract were corrected by these values. Figure 3 shows the plot of z-scores in ascending order. Laboratories, which are not considered, reported "below LOQ", or did not report any result.

Figure 3: Plot of z-scores for the spiked bread extract

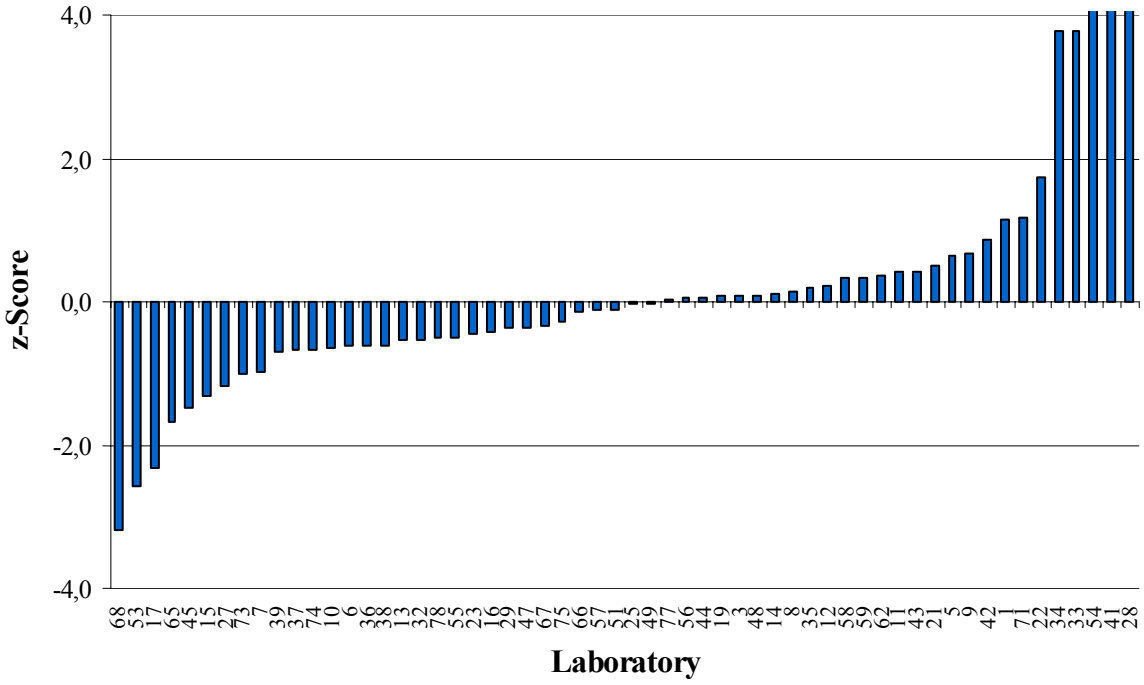

Table 3: Results of analysis and z-scores for the spiked bread extract; bold printed z-scores mark results outside the acceptable range

Laboratory	Reported result	z-Score	Laboratory	Reported result	z-Score	Laboratory	Reported result	z-Score
	µg/kg			µg/kg			µg/kg	
1	146.22	1.14	**27**	86.50	-1.18	**53**	51.00	**-2.56**
3	119.50	0.10	**28**	560.00	**17.21**	**54**	226.25	**4.24**
5	133.90	0.66	**29**	107.30	-0.38	**55**	104.18	-0.50
6	101.00	-0.62	**32**	103.50	-0.52	**56**	118.15	0.04
7	92.05	-0.97	**33**	214.50	**3.79**	**57**	113.95	-0.12
8	120.50	0.14	**34**	214.50	**3.79**	**58**	126.00	0.35
9	134.50	0.68	**35**	122.00	0.19	**59**	126.00	0.35
10	100.50	-0.64	**36**	101.10	-0.62	**62**	126.26	0.36
11	128.00	0.43	**37**	100.00	-0.66	**65**	74.00	-1.67
12	122.65	0.22	**38**	101.25	-0.61	**66**	113.42	-0.14
13	103.00	-0.54	**39**	99.00	-0.70	**67**	108.20	-0.34
14	120.00	0.12	**41**	400.00	**10.99**	**68**	35.05	**-3.18**
15	83.45	-1.30	**42**	139.50	0.87	**70**	111.00	-0.23
16	106.00	-0.43	**43**	128.00	0.43	**71**	147.00	1.17
17	57.00	**-2.33**	**44**	118.60	0.06	**73**	91.00	-1.01
19	119.20	0.09	**45**	79.00	-1.48	**74**	100.00	-0.66
21	130.05	0.51	**47**	107.50	-0.37	**75**	110.00	-0.27
22	162.00	1.75	**48**	119.50	0.10	**76**	112.50	-0.17
23	105.25	-0.46	**49**	116.50	-0.02	**77**	117.50	0.02
25	116.00	-0.04	**51**	114.36	-0.10	**78**	103.80	-0.51

Evaluation of the analysis data for the AA standard solutions

Overview

An aqueous AA solution and an AA standard in EtAc were prepared by the metrological division of IRMM. The concentrations of the standard solutions were adjusted according to the enrichment factors of the methods they were prepared for. The aqueous standard (48.2 ng/mL) was sent to laboratories that applied LC/MS/MS, LC/MS, LC/LC/DAD and GC/MS including derivatisation of AA. The standard in the organic solvent (149.5 ng/mL) was meant for laboratories that determine the AA content of food samples by GC/MS without prior derivatisation of AA. Due to the different chemical nature of the internal standards that are applied from the participants, an internal standard was not added to the standard solutions.

A number of 39 analysis results were considered in the data evaluation of the aqueous AA solution and 17 in the evaluation of the organic standard. Four laboratories did not report results.

Results

Instead of calculating z-scores, the percentage of the deviation of the reported values from the calculated AA content of the standard solutions was determined. The respective values for the aqueous solution are listed in table 4, whereas those for the standard in EtAc are shown in table 5.

Table 4: Aqueous standard: Results of analysis and deviation from calculated AA content

Laboratory	Reported result	Deviation	Laboratory	Reported result	Deviation	Laboratory	Reported result	Deviation
	µg/kg	%		µg/kg	%		µg/kg	%
1	54.38	12.75	**19**	47.50	-1.51	**48**	48.10	-0.27
3	48.55	0.66	**22**	62.50	29.59	**49**	49.40	2.43
5	55.50	15.07	**23**	34.05	-29.40	**51**	50.02	3.71
7	49.65	2.94	**25**	51.00	5.74	**56**	46.35	-3.90
8	48.05	-0.37	**28**	855.00	1672.76	**57**	47.45	-1.62
9	51.78	7.35	**32**	30.50	-36.76	**58**	49.50	2.63
10	58.50	21.29	**34**	40.00	-17.06	**62**	43.15	-10.54
11	46.25	-4.11	**36**	39.30	-18.52	**65**	50.50	4.71
13	43.00	-10.84	**38**	41.75	-13.44	**66**	41.89	-13.16
14	67.00	38.92	**39**	39.50	-18.10	**70**	95.00	96.97
15	39.85	-17.38	**41**	78.00	61.73	**73**	48.00	-0.48
16	96.00	99.05	**42**	77.50	60.69	**75**	87.00	80.39
17	44.00	-8.77	**44**	51.70	7.19	**78**	48.90	1.39

Table 5: Organic standard: Results of analysis and deviation from calculated AA content

Laboratory	Reported result	Deviation
	µg/kg	%
6	130.00	-13.06
12	177.25	18.55
27	124.65	-16.63
29	123.60	-17.34
33	110.00	-26.43
35	98.50	-34.12
37	100.00	-33.12
45	142.00	-5.03
47	173.00	15.70
53	110.00	-26.43
54	121.60	-18.67
55	100.50	-32.78
59	135.50	-9.38
67	179.00	19.72
68	125.00	-16.40
74	170.20	13.83
77	152.50	1.99

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Annex 1: Application form

Questionnaire for the participation in an European interlaboratory comparison study on the analysis of acrylamide from butter cookies and crispbread

Laboratory name:	
Contact person: Mr. ☐ Mrs. ☐	
First name:	Family name:
E-mail:	
Address: Street:	
Zip-code:	City:
Country:	Tel.:

Analysis method:	**LC/MS/MS** ☐ **LC/MS** ☐ **GC/MS (derivatisation)** ☐ **GC/MS (without derivatisation)** ☐
Extraction: **Extraction technique**	Orbital shaker ☐ Horizontal shaker ☐ Blender ☐ Ultrasonic bath ☐ Ultra turrax ☐ Vortex ☐ Stirrer ☐ Shaking water bath ☐ ASE ☐ Maceration ☐ Other ☐ (specify):
Extraction solvent:	Water ☐ Water/organic ☐ Organic ☐
Clean-up	SPE ☐ which cartridge(s): Carrez ☐ Liquid/liquid extraction ☐ Other ☐ (specify):
Quantification	Internal standardisation ☐ / external standardisation ☐
Internal standard	D_3-acrylamide ☐ $^{13}C_3$-acrylamide ☐ $^{13}C_1$-acrylamide ☐ Methacrylamide ☐ Propionamide ☐ N,N-dimethylacrylamide ☐
Experience in acrylamide analysis	High ☐ Medium ☐ Low ☐
Total analysis time (after delivery of samples)	1 week ☐ 2 weeks ☐ 3 weeks ☐ more than 3 weeks ☐

Please return questionnaire to: **e-mail: thomas.wenzl@irmm.jrc.be**
or fax: +32-14 571 343

Deadline: 25th May 2003

Annex 2: Sample receipt form

Interlaboratory comparison test on the anlysis of acrylamide from butter cookies and crisp bread samples

COLLABORATIVE STUDY MATERIALS RECEIPT FORM

Name of Participant	
Affilation	

Please ensure that the items listed below have been received undamaged, and then describe the relevant statement:

Date of the receipt	
All items have been received undamaged	Yes ☐ / No ☐
Items are missing/damaged.	Yes ☐ / No ☐

Contents of parcel

a) Collaborative trial materials coded as butter cookies and crisp bread (at least 1 package of 50 g each)

b) At least one amber vial identified as "Raw extract"

c) At least one amber vial identified as "Raw extract spiked with acrylamide"

d) At least one amber vial identified as "Acrylamide standard"

e) One sample description form

Please return the completed form to: Thomas.Wenzl@irmm.jrc.be

Annex 3: Analysis results report form

Spreadsheet for the transmission of analysis data

(For the interlaboratory comparison test on the analysis of acrylamide in butter cookies and crispbread samples)

Laboratory name	Address	Name of analyst	Date of analysis	Applied measurement technique (e.g. LC/MS/MS)	Sample name	Sample code	LOD	LOQ	First determination		Second determination		
									Weight-in quantity	Result	Weight-in quantity	Result	Mean
							µg/kg	µg/kg	g	µg/kg	g	µg/kg	µg/kg
					Crispbread								0,00
					Butter cookies								0,00
							ng/mL	ng/mL	mL (or g)	ng/mL	mL (or g)	ng/mL	ng/mL
					Blank extract	—							0,00
					Spiked extract	—							0,00
					Standard solution	—							0,00

Please use "." instead of "," for figures

If values are below LOQ, please give half of the LOQ value instead of using "<" sign (e.g. LOQ=70 µg/kg report 35 instead of <70)

If you use more than one measurement technique, please fill out separate spreadsheets for each technique.

Please return spreadsheet by e-mail to: Thomas.Wenzl@irmm.jrc.be

Annex 4: Homogeneity data

Table 1: Homogeneity data for the crispbread sample

sample id	**acrylamide (µg/kg)**	
	replicate 1	replicate 2
1	46	45
2	42	55
3	54	41
4	42	51
5	44	61
6	52	53
7	47	50
8	53	42
9	43	44
10	59	52
mean	48.81	

ref. for σ	Horwitz
target σ	12.3
s_a	6.7
F	0.83
F critical	3.02
F<Fcrit?	PASS
s_s	
s_s/σ	
critical s_s/σ	0.3
s_s/σ<critical s_s/σ?	

Table 2: Homogeneity data for the butter cookies sample

sample id	**acrylamide (µg/kg)**	
	replicate 1	replicate 2
1	160	142
2	151	150
3	152	154
4	149	159
5	1154	154
6	150	166
7	161	156
8	149	146
9	176	153
10	135	154
mean	153.55	

ref. for σ	Horwitz
target σ	32.55
s_a	9.0
F	0,83
F critical	3.02
F<Fcrit?	PASS
s_s	
s_s/σ	
critical s_s/σ	0.3
s_s/σ<critical s_s/σ?	

Table 3: Homogeneity data for the spiked bread extract

sample id	**acrylamide (µg/kg)**	
	replicate 1	replicate 2
1	125	117
2	120	115
3	116	113
4	118	121
5	117	114
6	114	116
7	117	116
8	122	123
9	117	122
10	115	117
mean	117.75	

ref. for σ	Horwitz
target σ	28.90
s_a	2.7
F	1.97
F critical	3.02
F<Fcrit?	PASS
s_s	
s_s/σ	
critical s_s/σ	0.3
s_s/σ<critical s_s/σ?	

Annex 5: Analytical methods used by participants

The method details are tabulated as they were reported by the participants. Not tabulated information was not submitted.

General methodology																						
LC/MS/MS	3	7	8	9	10	11	22	23	34	36	40	41	44	48	49	51	57	62	66	70	73	75
LC/MS	1	15	17	18	19	58																
LC/MS with derivatisation	12																					
LC/LC/DAD	56																					
GC/MS with bromination	5	16	28	32	39	43	65	78														
GC/MS without derivatisation	21	27	29	37	45	53	54	55	59	67	68	71	74	76	77							
GC/MS/MS	6																					
Sample weight, g																						
0,0-1,0	7	8	9	10	15	22	37	66	74													
1,01-3,0	5	34	62	67																		
3,01-5,0	1	3	11	12	17	19	21	36	40	44	45	48	49	51	56	57	58	59	68	71	73	
5,01-10,0	6	18	23	27	41	43	53	54	65	78												
10,0-15,0	16	28	32	39	55	77																
>15	29	70	75	76																		
Internal Standard																						
d_3-AA	3	5	8	9	10	11	12	16	17	19	21	22	23	27	32	34	36	41	44	45	48	49
	51	54	55	57	58	59	62	65	66	67	68	71	73	75	77	78						
$^{13}C_3$-AA	1	15	18	28	39	40	43	74														
Methacrylamide	27	28	37	53	68	75																
Propionamide	6	29																				
Butyramide	77																					
no internal standard	7	56																				

Extraction

Extraction solvent	
Water	1 3 5 6 7 9 10 11 15 16 18 19 22 23 27 28 29 32 34 36 39 40 43 48 49 51 53 54 56 57 58 59 62 66 71 78
Water / 1-propanol	12 (22/78) 55
Water / acetonitrile	21 (15/85) 67 (15/85) 17 (15/85)
Water / methanol	37 (10/90) 74 (10/90) 44 (95/5) 8 (95/5)
1-propanol	41 45 68 75
Ethyl acetate	28
other	65 (0,5% acetic acid)

Extraction solvent volumn, mL	
1,0	37 74
1,1 - 10,0	7 10 15 22 66
10,1 - 20,0	8 34 40 57 59 62 68 73
20,1 - 30,0	11 41
30,1 - 40,0	1 5 9 17 36 48
40,1 - 50,0	19 27 45 55 58 65 67 71 75 77
60	43
80	6 18 78
80,1 - 90,0	12 23
100	3 32 39 44 49 51 53 54 56
150	21
200	16 28 29

Extraction temperature	
Room temperature	5 11 28 36 37 43 44 48 58 66 68 73 74 78
20 °C	8 15 19 22 39 40 59
25 °C	10 12 18 21 67 75
37 °C	65
40 °C	3 9 17 41 51 57
50 °C	54
60 °C	6 29 34 45 53 55 56 62 71
65 °C	1
68 °C	27
70 °C	77
75 °C	49
80 °C	7 16 23 32

Maceration (time, min)	77 8 (30) 32 (120) 68 (30)

Addition of amylase	6 16 56 78

Extraction time, min																						
≤ 1	36	43	68																			
2 - 5	8	19	22	48	67																	
10	3	5	9	28	51	57	74															
16 - 20	10	49	66																			
25	15																					
30	6	12	18	21	27	29	34	37	39	40	53	56	58	59	62	71	77					
45	41	17																				
60	7	11	16	44	54	55	75															
90	1																					
120	23	32	78																			
≤ 960	65	73																				
others	45 (3 x 4 min																					
Extraction support																						
Shaker	8	10	11	15	39	40	41	66	73	75												
Ultrasonic bath	3	6	9	12	18	21	34	37	41	49	53	55	57	58	59	62	68	71	74	75		
Ultra Turrax	6	12	18	21	22	28	36	39	40	43	48	51	59	67								
Vortex	10																					
Stirrer	23	49	54	56	65	78																
Blender	7	29																				
Shaking water bath	1	5	7	55																		
ASE	17	45																				

Clean-up

Defatting	
Hexane	45 29 5 59 7 68 77 27 55 49 34 62 41 18 12 21 6 75
Dichloromethane	1 40
Petroleum ether	71
Solvent mixtures	57 (cyclohexane/butyl methyl ether = 95/5)
	53 (hexane/tert-butyl methyl ether)
	3 (isohexane/tert-butyl methyl ether = 95/5)
	51 (isooctane/butyl methyl ether = 95/5)
other methods	28 (1g Carbograph)
Carrez precipitation	3 9 16 21 32 34 49 51 53 56 62 65 67 71
Solid phase extraction	
IS MM 300mg / 3ccm	11 19 36 48 58
SEP-pak C18, 360 mg	73
Bond Elut Accucat 200mg / 3ccm	10 15 40
OASIS HLB 200mg / 6mL	10 15 22 40 66
M6N ABC 18	3
Isolute MFC18	9 51
Charcoal/alumina	19
Extrelute NT20	6 19 21 43 75
Liquid/liquid extraction	5 8 16 18 27 28 29 34 39 45 53 54 55 59 62 65 68 71 73 74 77
No special clean-up	7 17 44
Filtration	1 6 7 8 10 15 16 17 23 34 48 49 56 65 73
Ultrafiltration	1 11 22 36 48 58

LC methods

Column																							
C18 phase, not specified	7																						
Aquasil C18	40																						
Prevail C18	8																						
µ-Bondapak C18	10																						
Luna C18	17	49	51	75																			
Atlantis dC18	66																						
Optisphere HDO C18	15																						
Synergi Hydro RP	19																						
Lichrosphere C18	56																						
Excell ODS1	1																						
Hypercarb	3	9	11	22	23	36	48	57	58	73	75												
Luna Phenyl Hexxyl	18																						
Lichrosphere 100 CN	34																						
Aminex HPX-87H	56																						
Column lenght, mm																							
50	22	23																					
100	3	8	9	11	36	48	57	58	62														
150	7	17	44	49	51	66	75	75															
200	1	73																					
250	15	18	19	34	40	41	56																
300	10	56																					
Column internal diameter, mm																							
2,0	3	11	15	17	62	75																	
2,1	8	9	22	23	36	40	48	66	75														
3,0	1	41	49	51	57																		
3,2	44																						
3,9	10																						
4,0	34	56																					
4,6	7	18	19	58	73																		
7,0	56																						
Particle size, µm																							
3	8	49	51	66	75																		
5	3	9	11	18	22	23	34	36	44	48	57	58	75										
7	73																						
10	10																						

Mobile phase composition																							
Water	9	10	22	48	58																		
Water / methanol	1	11	15	19	23	40	41	49	57	66	73												
Water / acetonitrile	3	7	8	18	34	36	44	51	56	62	75												
Formic acid	3	8	9	15	23	44	49	51	57	62	73	75											
Acetic acid	1	7	9	10	18	34	48	66															
Ammonium formate	40																						
Sulfuric acid	56																						
Elution																							
Isocratic	1	3	7	8	9	10	11	15	19	22	36	40	41	44	48	49	51	56	57	58	62	66	
	75																						
Gradient	18	23	34	73																			
Column flow rate, µL																							
0,20	8																						
0,15	66																						
0,18	40																						
0,20	1	3	9	11	15	17	22	23	36	51	62	75											
0,25	41	49	57																				
0,30	44	73																					
0,40	48																						
0,50	19																						
0,60	10	56																					
0,70	34																						
0,80	18	80																					
1,00	7																						
Column temperature, °C																							
Room temperature	7	10	48	56	56	58	66																
25	34	62																					
26	11	15																					
28	40																						
30	3	9	19	23	44	51	75																
32	1																						
35	17																						
40	36	73																					
50	8																						
55	56																						
60	41	49	57																				

Injection volumn, µL																							
5	44																						
10	11	15	23	40	41	48																	
20	3	8	9	18	22	49	51	62	66	75													
25	17	73																					
40	34																						
50	1	7	19	36	57																		
100	10	58																					
500	56																						
Ionisation technique																							
APCI	66	10	41	51	34	19																	
ESI+	1	3	7	8	9	10	11	15	17	18	22	23	34	36	40	41	44	48	49	51	57		
	62	66	73	75																			
Recorded ions																							
72>72	10	66																					
72>55	3	7	8	9	10	11	19	22	23	34	36	40	41	44	48	49	51	57	62	66	73	75	
72>54	3	19	40	49	51	57	62																
72>44	3	10	34	36	40	44	57																
72>27	10	40																					
75>58	8	11	22	23	36	40	44	48	49	57	62	66	75										
75>57	73																						
75	19																						
72	15	19	58																				
55	19																						
54	19																						
72.044, 75.06, 51.02, 58.04 (TOF)	17																						
226, 229	18																						

GC methods

Column																						
BGB Wax	6																					
BPX-50	29	39																				
Carbowax	77																					
DB WaxETR	37																					
DB17	43																					
DB-1701	78																					
DB-17MS	16																					
DB-5MS	5																					
DB-Wax	55																					
FFAP	12	21	53	68																		
HP 5MS	32																					
HP-5	28																					
Innowax	27	59	71	74																		
Solgelwax	45																					
SUWAX 10	54																					
ZB5	65	67																				
Column lenght, m																						
10	77																					
25	59																					
30	5	6	12	16	21	27	28	29	32	37	39	43	53	55	65	67	68					
60	45	54	71	74	78																	
Column internal diameter, mm																						
0,2	59																					
0,25	5	6	16	27	28	32	37	39	43	45	54	55	65	67	68	71	74	77	78			
0,32	12	21	29																			
Thickness of stationary phase, µm																						
0,25	5	6	12	16	21	27	28	32	37	39	54	55	65	68	71	74	78					
0,30	45																					
0,40	59	77																				
0,50	29																					
1,00	67																					
5,00	43																					

Temperature programm, °C	
65 isothermal	43
60/1-12-210/0-50-230/4	6
65/2-10-280/0	39
80/2-8-250/0	37
55/2-25-175/6-50-280/6	16
65/1-15-250/10	5
60/1-10-200/3-30-230/5	55
50/3-50-240/9	68
65/1-15-185/0-20-280/0	32
65/1-6-145/0-25-250/0	28
80/1-15-220/4	27
50/0,1-20-70/0-8-270	65
50/2-5-250/0-30-280/3	78
60/2-15-240/11	71
60/1-12-230/10	74
70/2-10-250/5	54
60/2-10-240/10	21
60/2-10-240/10	12
70/2-20-220/0-6-270/5	45
80/5-10-200/5-20-240/10	59
70/2-15-220/2	77
80/0-10-200/0-25-250/2	29
70/1-4-150/6-100-200	53

Injection volumn, µL	
1	6 28 29 37 43 45 54 55 68 74
2	5 12 16 21 27 39 53 65 67 71 78
3	32 59 77

Injection technique	
split	37
splitless	68 28 74 54 45 29 5 39 16 27 53 59 32
PTV	55 65 78 67
on-column	21 12 77 6

Ionisation technique	
EI	37 68 28 5 39 16 53 59 32 65 78 67 43 71
PCI	74 54 45 77 29 6 21 12 27
NCI	55

Recorded ions, m/z	
74>55	6
72>55	6
41	68
44	68
47	68
55	29 37 59 67 68 71
58	59 67 68 71
69	37 68
70	55
71	29 37 53 59 67 68 71
72	12 21 27 29 45 54 74 77
73	55
74	59 67 68 71
75	12 21 27 54 74 77
85	37 68
86	45 77
89	45 54 74 77
92	45 54 74
103	45
106	5 32 65 78
108	5 32 43 78
109	5 43 65
110	78
111	5
133	39
138	39
149	39 78
150	5 16 28 32 43
151	78
152	5 16 32
153	5 16 32 43 78
154	39
155	16 28 32
156	5
180	28